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SCIENTIFIC MEMOIRS

BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA.

ON THE STANDARDISATION OF ANTI-TYPHOID VACCINE.

BY

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(Director, Pasteur Institute of India),

AND

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ON THE STANDARDISATION OF ANTI-TYPHOID VACCINE.

FEW will deny that vaccination against typhoid fever has now taken its place amongst the measures of preventive medicine. This achievement is principally due to the brilliant work and the untiring exertions of Wright, who since 1897 has devoted so much time and energy to the various problems which surround this subject. That success has crowned his efforts is certain from the facts, (1) that three scientific committees, successively convoked to examine into the matter, have decided that the statistical evidence is sufficient to show that the incidence and case mortality are reduced by inoculation, and (2) that the Germans have adopted it in their army in the case of troops proceeding to foreign service in Africa. There are still, however, many problems to be solved before anti-typhoid vaccination becomes generally adopted by all who practise preventive medicine, and one of the most important of these problems is the elaboration of some method of standardisation. Before we consider the various methods of standardisation which have been employed heretofore, it will be necessary to glance for a moment at the nature and constitution of the vaccines which are in common use.

An anti-typhoid vaccine may be prepared in any of the following ways:—

(*a*) Sterilising by heat or by some antiseptic, such as lysol or carbolic acid, a well-grown broth culture of bacillus typhosus. This method has been greatly used by Wright and is, we believe, the method still employed by him. A vaccine of this nature was employed by Leishman, Harrison, Smallman and Tulloch in a recent research[1] on the blood changes following anti-typhoid inoculation. We shall call this 'broth vaccine'.

Vaccines prepared by this method consist of:—

(1) Constituents of the broth, such as peptone, etc.

(2) The extra-cellular toxins of the bacteria.

(3) The free "receptors" arising either by disassociation from, or by the dissolution of, the bodies of the bacteria.

(4) The undissolved bodies of dead bacteria with their intra-cellular toxins and constituent receptors.

(*b*) Suspending the surface growth of agar cultures of bacillus typhosus in a fluid medium, such as normal salt solution, and then killing the bacteria in the same way as in the case of broth cultures.

This was the original method described by Wright[2] in 1897. It is the method now recommended by a committee of German bacteriologists convoked at the instance of the Prussian Government[3]. Such a vaccine we propose to call 'agar vaccine'.

An agar vaccine is of similar composition to a broth vaccine, the constituents of the broth and the extra-cellular toxins being, however, in very small quantity.

(*c*) Dissolving up the bodies of the bacteria, either in a broth culture or in the emulsions of the agar cultures, and then filtering through a porcelain filter. It was first shown by Wright and Windsor[4] that the filtrate of a broth culture of bacillus typhosus, which had been macerating at 37°C. for a period of five months, diminished the bactericidal power of a serum with which it was mixed to exactly the same degree as the unfiltered culture. These workers did not suggest the use of such a filtrate as a vaccine.

Strong[5] working with the cholera vibrio prepared a vaccine in the following manner:—

The surface growths on agar, 20 hours old, were suspended in sterile water; the bacteria were then killed by heating at 60°C. for from one to twenty-four hours; the mixture was afterwards put aside in the incubator at 37°C. for from two to five days and finally filtered through a Reichel candle. With such a vaccine he was able to immunise animals and to produce both specific agglutinins and bactericidal substances in their blood. A vaccine prepared in this manner was also used in a few instances on the human subject with results similar to those which were obtained on animals.

A similar method of preparation has been adopted in the case of anti-typhoid vaccine by Bassenge and Mayer[6]. These workers suspended typhoid bacteria grown on agar in sterile water; the emulsion was well shaken up and then filtered. By this means a filtrate, free from bacteria and which gave rise on inoculation into animals and men to agglutinating and bactericidal substances, was obtained. A vaccine prepared by this method we shall designate 'filtrate vaccine', the word 'agar' or 'broth' being prefixed according as an agar or a broth culture has been used in its preparation.

A filtrate vaccine will be of the same composition as the broth or agar vaccine from which it was prepared, less the bodies of the bacteria and particulate debris, the remains of broken-up organisms. If such a vaccine is properly prepared, the total receptors which are present in the original emulsion before filtration should be found in the filtrate.

Many advantages have been claimed for these filtrate vaccines, to wit, that being clear and free from suspension their freedom from contamination is easily appreciable even by a layman; that the local and general reactions caused by their injection is much lessened; and finally that the degree of immunity

conferred is as great as that given by other vaccines which contain the bodies of the bacteria and lasts as long.

Other methods of preparing anti-typhoid vaccine, such as that described by Macfadyan and Rowland, have been promulgated from time to time, but as far as we know all vaccines at present in common use are obtained by one of the methods described above.

With this introduction we may now pass on to the question of the standardisation of these several vaccines, a question which is generally recognised to be of dominating importance. The first desideratum in this connection is of course to place before ourselves a perfectly clear mental picture of what is connoted by the term standardisation. It seems to us that Wright[7] has supplied us with an excellent definition of the term. He says, 'Standardisation involves, on the one hand, the determination of the amount of immunising element contained in a standard volume of the vaccine material; on the other hand, the adjustment of the dose administered to the resisting powers of the man or animal subjected to the inoculation.' If we accept this definition, it is evident that the first problems which have to be settled before any scientific method of standardisation can be evolved are problems concerning the nature of the immunising element of the vaccine and the manner in which it accomplishes its object. Let us for a moment consider these questions.

It will, we think, be acknowledged by all pathologists that the immunity induced by anti-typhoid vaccination is in the main, if not entirely, a bactericidal immunity, that is to say, that it depends on an increased power of the individual to kill the typhoid bacteria by means of his body fluids, and that there is no evidence so far of an antitoxic immunity. This increased power is due, using the language of Ehrlich, to an increase of the specific amboceptor or immune body in the body fluids and not to any increase of the complement.

It is also probable that the organism, after an anti-typhoid inoculation, acquires a habit of producing these protective substances on a stimulus of a much slighter nature than before the inoculation. Such an hypothesis would account for the presence of a certain degree of immunity after the amount of protective substances, which are increased considerably in quantity immediately after an inoculation of anti-typhoid vaccine, had again fallen to normal.

It is, however, possible that we have also to deal with the action of the substances called by Wright 'opsonins' or of the substances named by Metchnikoff 'stimulins'. But the proof of these substances taking any part in the process of typhoid immunity is still lacking. We need not, therefore, take them into account at the present moment. We have consequently only to account for an increased production of immune body which is called forth as a result of the inoculation of the immunising substances contained in the vaccine.

Now, it has been proved that, if a considerable quantity of immunising substances be injected into an animal, this increase of immune body is preceded by a diminution called by Wright the 'negative phase'[8]. This diminution must be due to a union of the receptors of the immunising substances and the immune body. The mental picture, therefore, which we have formed of the process is as follows:—

First, a union of the total receptors injected with a certain proportion of the immune body normally present in the plasma; as a result of this fixation of immune body a disturbance of the equilibrium between the free amboceptors and those belonging to the protoplasmic molecule; then a casting-off of fixed amboceptors to take the place of those lost; and finally an over-production of these bodies. If this hypothesis is correct then it follows that the power of any vaccine to increase the quantity of immune body in the body fluids depends upon the number, and avidity for amboceptors, of the receptors contained in an unit quantity of the vaccine. The greater the number and the greater the avidity of the receptors, the greater will be the power of the vaccine to fix immune body; the greater the power of the vaccine to fix immune body, the more immune body will be fixed; the more immune body fixed the more will be reproduced; the more immune body reproduced the greater will be the resulting immunity. It appears to us, then, that the problem of determining the optimum dose of an anti-typhoid vaccine will only be solved when we are able to estimate that proportion of free immune body which can be fixed by the receptors of the vaccine without danger to the organism. The first step towards this end will evidently be a method of measuring the receptor content of any vaccine and this, we take it, is the true basis of standardisation of all anti-tyhoid vaccines, no matter how prepared. Let us for a moment consider in this light the various methods of standardisation which have been used heretofore.

These methods only apply to the broth and agar vaccines, in which it is assumed that a constituent of the bacterial protoplasm constitutes the essential immunising element, and therefore the amount of bacterial protoplasm in the vaccine will come into consideration in appraising the potency of the vaccine.

In his earliest inoculations Wright[9] used as a dose the amount of growth which covered a 1/20 to a 1/4 of an agar slope, the virulence of the culture being fixed. We have in this method at the very beginning an attempt, although a crude one, to estimate the amount of bacterial protoplasm injected.

At a later date Wright and Leishman[10] using broth vaccines determined the degree of opacity of each sample of vaccine, taking the opacity as a criterion of the number of undissolved bacteria which were contained in the unit of volume. At the same time cognisance was taken of the toxicity of the vaccine when injected into guinea-pigs.

The next step in the direction of estimating the amount of bacterial protoplasm was made by Wright[11] when he introduced the blood counting method of estimating both the living and dead germs in a unit volume of vaccine. Leishman and his colleagues[12] were unable to obtain consistently satisfactory results by this method. They found that in the case of broth vaccines errors of from 50 to 100 per cent. in counts of the same film, made by different observers, were by no means uncommon.

Harrison[13] has recently introduced a modification of Wright's method for counting the bacteria contained in a culture. In this worker's hands it has given better results than any method he has tried, and it seemed quite possible to keep the error below $\pm$ 10 per cent. in every case.

Finally[14] the amount of bacterial protoplasm in a vaccine has been estimated by weighing the dried bacterial bodies in a measured quantity of vaccine. This method has not, as far as we know, actually been used in standardising anti-typhoid vaccine, but it has been suggested as likely to be of great help in estimating the strength of a vaccine.

All these methods of standardisation aim at an estimation, as accurate as possible, of the bacterial protoplasm contained in a unit of vaccine. They, therefore, are quite inapplicable to the standardisation of 'filtrate vaccines.' Further, if our ultimate aim is a determination of the receptor content of a vaccine, these methods all fall short of an accurate estimate, inasmuch as they take no cognisance of those receptors which are free in the vaccine either as a result of their disassociation from the bacteria or as a result of the dissolution of the bodies of dead germs. Probably this objection might not hold good in the case of agar vaccines or in the case of broth vaccines prepared from young cultures, that is, 24 to 48 hours old, if the enumeration or weighment be made before any receptors have become free in the vaccine fluid. It would, however, with our present methods of experimentation be impossible to arrive at any estimate of when this takes place. It appears to us that we must assume that, since bacteria in cultures are constantly dying, receptors will be constantly being set free into the fluid medium.

Another objection which can be raised to all these methods of standardisation is that they take no cognisance of the virulence of the organism, the cultures of which are used in the preparation of the vaccines. Now, working with the *vibrio choleræ* Pfeiffer and Friedberger[15] showed that in the case of killed cultures of this organism the immunising effects were proportional to the virulence of the inoculated strain. They concluded that the virulent and the avirulent organisms differ in the number or degree of affinity of their haptophore groups; they demonstrated this conclusion by experiments in which it was shown that a virulent organism bound many more times the number of amboceptors than certain avirulent ones.

Strong [16] has recently confirmed and extended the work of Pfeiffer and Friedberger. This observer in a research which had as its object the determination of the exact relationship between the virulence of an organism and its power of binding amboceptors and of producing immunity arrived at the following conclusions:—

The virulent cholera spirillum possesses a greater number of bacteriolytic haptophore groups, or these groups are endowed with a greater binding power for the amboceptors than the avirulent organism; the number or the avidity of the bacteriolytic receptors possessed by a bacterium is directly proportional to its virulence; the binding power of the free receptors of the organisms for bacteriolytic amboceptors 'in vitro' is proportional to the bactericidal immunity produced in animals by each, which latter is in turn proportional to the virulence of the organisms from which the receptors were extracted; the bactericidal immunity obtained by means of the inoculation with the dead organisms of different virulence or their extracts (obtained by autolytic digestion) is proportional to the virulence of the living strains of the bacteria employed.

It is evident, therefore, that the virulence of the organism used in the preparation of a vaccine is of the greatest importance and must be taken into account in any method of standardisation. Further, it would appear that as virulence is in direct proportion to the number, or avidity for immune body, of the receptors, an estimation of these latter in any vaccine will take cognisance of the virulence of the organism from which it was prepared. If, therefore, we are able to determine the absolute receptor content of a vaccine, we shall have complied with the first proposition of the definition of standardisation which we have set before us.

Dr. Wright [17] was the first to suggest that anti-typhoid vaccines might possibly be standardised on this principle. He showed that these vaccines are capable of exerting an anti-bactericidal effect on a serum normally bactericidal to typhoid bacteria, and suggested that by progressive dilution of the vaccine this fact might be made use of to standardise vaccines. He did not, however, persevere with the working out of this suggestion, and his original methods of experiment could never have been used for the absolute standardisation of vaccines. To devise a technique for such an estimation would not be difficult, in fact we have already done so. But when we came to consider the second proposition of Wright's definition of standardisation, namely, the adjustment of the dose administered to the resisting powers of the man or the animal subjected to the inoculation, we saw that a better and easier method, a method of relative standardisation, which would answer all practical purposes, could be devised. While reserving for a future communication the application of this principle to the problem of the absolute standardisation, we propose now to deal fully with its application to the problem of the relative standardisation of anti-typhoid vaccines.

If the dose of vaccine administered is to be adjusted to the resisting powers each individual inoculated, this would involve a careful determination of the amboceptor content of the individual's serum on several occasions before the inoculation. Such an investigation is of course impossible when a large body of men is to be inoculated, but an average, such as has been done recently by Leishman and his collaborateurs,[18] can always be obtained, by the system devised by them of pooling equal measures of the different sera and testing the bactericidal power of this mixture. As we are dealing here only with an average, this determination may be ignored in practice.

But on further consideration of this proposition it is at once clear that the adjustment of the dose of any anti-typhoid vaccine to the average resisting powers of man, that is to say, the determination of the proper proportion of immunising element to the average amboceptor content of human plasma, which should be injected so as to give rise to the best immunity, can only be estimated by direct experiment. Such an experiment has been begun and is still in progress under the direction of Lt.-Col. Leishman.[19] Groups of men have been inoculated with different doses of an anti-typhoid vaccine, the constitution and bacterial protoplasm content of which have been carefully noted. The changes which these inoculations brought about in the blood have been thoroughly investigated during a period of four weeks after the first inoculation. While these original investigations have traced the origin and development of the protective substances and the immediate effects with different doses of vaccine, the further observations which are to be prosecuted in India, where the inoculated men have proceeded, will show us the fate of these substances and, what is of great importance, will give us an idea of the degree of immunity which has been conferred on each group of men. When this information has been obtained we shall be in a position to undertake the relative standardisation of anti-typhoid vaccines in general on the basis of the receptor content. For it is to be hoped that we shall then be in possession of a standard vaccine which will be available to all laboratories which manufacture anti-typhoid vaccine for the purpose of relative standardisation after the manner of the Ehrlich unit in the case of diphtheria antitoxin. Being provided with such a vaccine it will be the business of all to standardise against it every vaccine issued. We, therefore, propose to detail a method which we have elaborated with this end in view. This method is in reality an application of the principle upon which Wright's suggestion was based and consists of a comparison of the anti-bactericidal power which is exerted on a normal serum by the standard vaccine and those vaccines with which it is to be compared. Given a certain bactericidal serum we estimate what dilution of the various vaccines when mixed in equal parts with the serum is able to completely remove the bactericidal power of that serum, in other words, in what dilution of vaccine the receptors completely neutralise the amboceptor content of the serum.

The following is the detailed description of the technique.

The materials required are :—

(*1*) *Wright's diluting pipette.*—Any form of pipette constructed in accordance with the principles laid down by Dr. Wright[20] would serve. We have however, found it convenient to use a pipette in which the stem is graduated in ten divisions of five cubic millimetres each, the upper marks indicating 450 c. mm. and 500 c. mm. respectively.

(*2*) *A plentiful supply of sterile watch glasses.*—The watch glasses are sterilised in a covered copper vessel such as is used for the sterilisation of Petri dishes. When wanted they are removed with sterile forceps and placed in pairs one covering another.

(*3*) *Series of small test-tubes in racks.*—The best tubes are of small size. They are made from glass tubing of a diameter of 0·8 cm. ; they measure 4·5 cm. long ; a small bulb is blown at the end in order to increase the capacity without increasing the length.

They are plugged with wool and sterilised in the autoclave before use.

(*4*) *A 24-hour broth culture of bacillus typhosus.*—In order to secure a fairly constant strength of culture a broth culture should be prepared from day to day by planting into 10 cc. of broth 0·05 cc. of a broth culture of 24 hours' growth.

(*5*) *Normal serum bactericidal for bacillus typhosus.*—The serum which we have used throughout our experiments, and which we recommend, is that of normal goats. This serum exerts a marked bactericidal action on the typhoid bacillus, the degree of this action varying in different goats and also from day to day in the same goat.

The blood can be easily obtained from the jugular vein by means of a sterile syringe. It will, of course, depend upon the number of vaccines to be tested how much serum will be required, about 0·5 cc. of serum being used for each vaccine. The blood is allowed to clot in a sterile conical glass vessel covered with a lid. We have employed throughout our experiments only serum which had been in contact with the clot for 24 hours.

(*6*) *Vaccines to be tested.*—No antiseptic must be added to these before testing.

(*7*) *Sterile normal salt solution.*

(*8*) *Sterile broth.*

(*9*) *A beaker of sterile water*, which is kept constantly boiling. The pipette is easily and quickly sterilised when necessary by washing it out with this boiling water.

Having detailed our requirements in the way of apparatus, etc., we may now pass on to a description of the procedure.

For each vaccine a series of sterile covered watch glasses numbered from 1 to 10 is placed in front of the operator. Into glass No. 1 undiluted vaccine is placed. From this by means of the diluting pipette, which has been previously sterilised, different dilutions are prepared with normal salt solution, starting with a two-fold dilution and finishing with a ten-fold dilution.

In making this series of dilutions we have found it convenient, first to measure out the normal salt solution into the respective watch glasses, and then to add the requisite quantity of vaccine to each. The following table shows at a glance for each dilution the most convenient amounts of the two substances:—

No. of watch glass.	Dilution.	Normal salt solution.	Vaccine.
2	2 fold.	100 cm.	100 cm.
3 . .	3 "	100 "	50 "
4	4 "	150 "	50 "
5 . . .	5 "	200 "	50 "
6	6 "	250 "	50 "
7	7 "	300 "	50 "
8	8 "	350 "	50 "
9	9 "	400 "	50 "
10	10 "	450 "	50 "

These dilutions being made, the small test-tubes, numbered 1 to 10, are arranged in series in a rack. Into each tube there are measured carefully 100 cm. of the corresponding dilution of vaccine; thus No. 1 tube receives 100 cm. of undiluted vaccine, No. 2 tube 100 cm. of a two-fold dilution and so on.

The same process as described above is carried out in the case of each vaccine to be tested and, also, of course, in the case of the standard vaccine against which they are being tested. After this has been accomplished, into each tube there are measured 100 cm. of the fresh serum of the goat, the same serum being used for all tubes. The contents of the tubes are then well shaken up so as to procure complete mixture. During this operation the small bulb at the bottom of the tube prevents the soiling of the upper portion of the tube and of the cotton wool plug. All preparations are then placed in the hot-air chamber (37°C.) for one hour, each series of tubes being placed in a separate tin bearing the designation of the vaccine.

While the tubes are in the incubator we proceed to make ready the dilution of typhoid culture, a small quantum of which is to be added to each tube in order to test as to whether or not the total amboceptor content has been removed from the serum. For this purpose we have found it convenient to add to each tube 50 cm. of a 1,000,000-fold dilution of a 24-hour broth culture. If care be taken to prepare this culture in the manner we have mentioned above, it is possible

to obtain from day to day cultures containing approximately the same number of living bacteria. In a large number of experiments made to test this point we have found that such a culture contains on an average 400,000,000 living bacteria per c.c., when the enumeration was done in the manner described by Wright [21]. We can, therefore, calculate that 50 cm. of a 1,000,000-fold dilution of such a culture will on an average contain eight bacteria, a number eminently suitable to indicate whether or not the anti-bactericidal effect of the dilution of vaccine on the serum has been complete. We, therefore, proceed to prepare in sterile watch glasses and by means again of Wright's diluting pipette a 1,000,000-fold dilution of the typhoid culture, using sterile broth as the diluent. The actual amount of this dilution which must be made ready depends of course on the number of vaccines which are being tested. To each tube there is then added 50 cm. of the 1,000,000-fold dilution.

The tubes are then thoroughly shaken to insure an even distribution of the bacteria throughout the contents. They are again placed in the incubator at 37°C. and left there for from 18 to 24 hours. At the end of this time 0·5 cc. of sterile broth is added to each tube. They are again incubated, this time for three days. After this interval it is easy to see in which tubes a growth has taken place and in which the contents are sterile. If there be any doubt on this point, or if there be any irregularities, samples are planted on to agar and any resulting growth stained and examined in the usual way.

Before passing on to put forward some examples of this method of comparative standardisation we have to note several points which have to be carefully attended to in connection with the technique above described.

(1) The vaccine, unless it be a filtrate vaccine, must be thoroughly shaken up before testing so as to insure an even distribution of the suspended bacteria.

(2) After the serum has been added to the vaccine dilutions the tubes must be thoroughly shaken before being incubated. If the vaccine is one containing the bodies of the bacteria, it is well to shake the tubes once or twice during the period they are in the hot-air chamber.

(3) While adding the 50 cm. of the culture dilution to each tube great care must be taken to avoid soiling the sides of the tubes with the living bacteria. The end of the pipette should be carefully introduced, without touching the sides, to the very bottom of the tube before the 50 cm. are expelled. If this point is not consistently attended to, the most discordant results will be obtained. The sides of the tubes become soiled with living bacteria, on which the serum is not able to exert its bactericidal action and which, therefore, grow after the addition of the sterile broth.

(4) After the addition of the living bacteria all the tubes must be again thoroughly shaken before being incubated.

(5) At least four tubes containing only sterile broth should be inseminated with 50 cm. of the 1,000,000 dilution of the typhoid culture, in order to control the certain presence of living bacteria in the quantum added to each tube. In our experience, however, we have never found that any of these tubes remained sterile.

(6) In order to test the sterility of the vaccines, of the serum and of the salt solution samples of these should always be planted on agar or in broth.

(7) After the addition of the sterile broth it is necessary to incubate the tubes for three days, as owing to the inhibitory action of the serum growth may be considerably delayed.

As an example of the results which are to be obtained by this method, we have now to detail comparative observations made with two vaccines, one broth and one agar, and the sera of three different goats. The vaccines were prepared as follows:—

Vaccine I was a broth culture of bacillus typhosus which had been incubated at 37°C. for four weeks. It was killed by heating in a water bath at 60°C. for half an hour.

Vaccine II was prepared by emulsifying agar cultures, 48 hours old, with normal salt solution. No attempt was made to measure the amount of the salt solution used. The bacteria were killed by heating the emulsion at 60°C. for half an hour.

These two vaccines were tested side by side, after the manner already described, against the sera of three different goats.

The following were the results obtained:—

4th May 1905. Experiment I. Serum of goat IV.

Dilution of vaccine.	Vaccine I.	Vaccine II.
Undiluted. . . .	Growth	Growth.
2-fold dilution . .	,,	,,
3 ,, ,, . . .	Sterile	,,
4 ,, ,, . . .	,,	,,
5 ,, ,, . . .	,,	,,
6 ,, ,, . . .	,,	Sterile
7 ,, ,, . . .	,,	,,
8 ,, ,, . . .	,,	,,
9 ,, ,, . . .	,,	,,
10 ,, ,, . . .	,,	,,

It will be seen from this table that vaccine I in a two-fold dilution completely removed the bactericidal action of the serum, while in a three-fold dilution it failed to do so. We may, therefore, for purposes of calculation hereafter take it that "neutralisation" between receptor of vaccine and amboceptor of serum would be obtained when a dilution of 2·5 of vaccine was added in equal parts to the serum. On the other hand, the neutralisation point in the case of vaccine II and the serum would be obtained in a 5·5-fold dilution. Thus we see that, as regards receptor content, vaccine I stands in relation to vaccine II as 2·5 to 5·5, or as 1: 2·2. In other words, vaccine II is 2^1 times 'stronger' in immunising elements than vaccine I.

12th May 1905. Experiment II. Serum of goat V.

Dilution of vaccine.	Vaccine I.	Vaccine II.
Undiluted . . .	Growth.	Growth.
2-fold dilution . . .	Sterile.	"
3 " " . . .	"	"
4 " " . . .	"	Sterile.
5 " " . . .	"	"
6 " " . . .	"	"
7 " " . . .	"	"
8 " " . . .	"	"
9 " " . . .	"	"
10 " " . . .	"	"

In this experiment the serum was evidently richer in amboceptor content than was the case in the previous observation. Thus, the neutralisation point in the case of vaccine I would be obtained in 1·5-fold dilution and with vaccine II in a 3·5-fold dilution. Thus, the relation of their receptor contents would be as 1·5 to 3·5, or as 1 : 2·3. In other words, vaccine II

contains about $2\frac{1}{3}$ times the amount of immunising element that vaccine I contains.

19th May 1905. Experiment III. Serum of goat VII.

Dilution of vaccine.	Vaccine I.	Vaccine II.
Undiluted . . .	Growth.	Growth.
2-fold dilution . . .	"	"
3 " " . . .	"	"
4 " " . . .	Sterile.	"
5 " " . . .	"	"
6 " " . . .	"	"
7 " " . . .	"	"
8 " " . . .	"	"
9 " " . . .	"	Sterile.
10 " " . . .	Growth.	"

The growth in tube 10 of vaccine I was evidently an experimental error. It is, therefore, left out of account. It is clear that the serum in this observation was much less bactericidal than in either of the two experiments previously detailed. We can calculate, in the same way as we have done before, that vaccine II is $2\frac{1}{25}$ times stronger as regards its immunising power than vaccine I.

When we compare the results of these three observations made with the same two vaccines but with three different sera on three different days, we find that the relative strengths, as regards receptor content, were practically constant, namely, (1) 1 : 2·2, (2) 1 : 2·3, and (3) 1 : 2·4; the average of the three observations being 1 : 2·3. We can, therefore, take it that vaccine I as regards its immunising power is 2·3 times weaker than vaccine II and that the dose of the former should be 2·3 times greater than in the case of vaccine II. If the dose of vaccine II be 1 cc., then we would expect the same immunising effect from 2·3 cc. of vaccine I.

In conclusion, we have now to show that this method of comparative standardisation is also applicable to filtrate vaccines. Wright and Windsor[11] have already demonstrated that the filtrate of a broth culture of bacillus typhosus

which had been macerating before filtration at 37° C. for a period of five months, was able to diminish the bactericidal power of serum to the same degree as the original culture. In other words, the receptors of the typhoid bacteria after five months' maceration had become completely dissociated from the protoplasm molecule and were found free in the fluid part of the vaccine.

We now tested side by side, (1) a broth vaccine which had been grown for seven weeks before sterilisation and then allowed to macerate at 37° C. for a further period of four weeks before testing, and (2) the filtrate of this culture got by passing it through a Berkfield filter after the four weeks of maceration.

The following was the result:

Dilution of vaccine.	Broth vaccine.	Filtrate vaccine.
10-fold	Sterile.	Sterile.
9 "	"	"
8 "	"	"
7 "	"	"
6 "	"	"
5 "	"	"
4 "	"	"
3 "	"	"
2 "	Growth.	"
Undiluted	"	"
*Dilution of serum	"	"
2-fold	"	Growth.
3 "	"	"

* In the two last tubes of each series the diluted serum was mixed with an equal quantum of undiluted vaccine.

From this table it is seen that the broth vaccine possessed between three and four times the anti-bactericidal power of the filtrate vaccine. Maceration had not evidently been continued long enough to allow of the total receptors being completely set free from the bacteria.

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